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c-*fos* Is Required for Malignant Progression of Skin Tumors

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Summary

The proto-oncogene c-*fos* is a major nuclear target for signal transduction pathways involved in the regulation of cell growth, differentiation, and transformation. Using the multistep skin carcinogenesis model, we have directly tested the ability of c-*fos*-deficient mice to develop cancer. Upon treatment with a tumor promoter, c-*fos* knockout mice carrying a v-H-*ras* transgene were able to develop benign tumors with similar kinetics and relative incidence as wild-type animals. However, c-*fos*-deficient papillomas quickly became very dry and hyperkeratinized, taking on an elongated, horny appearance. While wild-type papillomas eventually progressed into malignant tumors, c-*fos*-deficient tumors failed to undergo malignant conversion. Experiments in which v-H-*ras*-expressing keratinocytes were grafted onto nude mice suggest that c-*fos*-deficient cells have an intrinsic defect that hinders tumorigenesis. These results demonstrate that a member of the AP-1 family of transcription factors is required for the development of a malignant tumor.

Introduction

Advances in molecular biology have served to outline the multiple genetic components that are involved in the development of cancer. It is now well established that for a cell to grow aggressively within the context of the organism, proto-oncogenes must be mutated to become active growth inducers, tumor suppressor genes must be functionally lost, and the cell must be released from its own self-destruction program. Many of the important molecules in cancer development are proteins that function outside the nucleus of the cell, either assessing changes in the extracellular environment or relaying messages from sensor proteins to the control center of the cell. Because mitogens, growth factors, and tumor promoters regularly use these signal transduction pathways, mutations in genes implicated in either signal recognition or propagation can induce the cell to behave inappropriately. Upon reaching the nucleus, signals from these proteins must communicate with transcription factors to elicit the changes in gene expression that can allow the cell to react to its new environment.

One class of transcription factors that it is thought to serve as the nuclear target of many oncogenic signal transduction pathways is the activator protein 1 (AP-1) family (reviewed by Ransone and Verma, 1990; Angel and Karin, 1991). This multigene group includes members of the Fos (c-*fos*, *fosB*, *fra-1*, and *fra-2*) and Jun (c-*jun*, *junB*, and *junD*) families. Members of the Fos family form heterodimers with Jun proteins and regulate transcription by binding DNA at AP-1 sites. These *cis*-acting regulatory elements are found in a variety of genes involved in cell proliferation, differentiation, and tumorigenesis. Oncogenic activation of several signal transduction pathways can result in increased AP-1 activity. For example, tumorigenic mutations in the proto-oncogene H-*ras* activate a family of mitogen-activated protein (MAP) kinases (JNKs/SAPKs) that phosphorylate Jun to augment its transactivation potential (Pulverer et al., 1991; Binetruy et al., 1991). Recently, an analogous class of Ras-responsive intracellular kinases has been described that seems to regulate Fos activity (Deng and Karin, 1994). It is unclear from these in vitro studies, however, how elevated AP-1-dependent gene expression can contribute to cancer development in vivo.

A clue to the significance of AP-1 activity for cell transformation can be found in the history of *fos* and *jun*, as both genes were initially described as the transforming principles of oncogenic retroviruses (Curran et al., 1982; Maki et al., 1987). Later studies using transgenic mice showed that stable expression of c-*fos* led to a dysregulation of bone growth eventually resulting in osteosarcomas and chondrosarcomas (Ruther et al., 1987, 1989). Transgenic mice expressing an oncogenic form of *jun* developed fibrosarcomas at sites of wound healing (Schuh et al., 1990). These observations indicated that aberrant expression of AP-1 genes could affect mitogenic control and promote neoplastic transformation of specific tissues.

Through the use of embryonic stem cell gene targeting technology, null mouse mutations of c-*fos* and c-*jun* have recently been generated (Johnson et al., 1992, 1993; Wang et al., 1992; Hilberg et al., 1993). While the c-*jun* knockout mutation is embryonic lethal, the c-*fos* null mouse is viable, though it displays a variety of tissue-specific anomalies. These defects include a severe form of osteopetrosis, a mild lymphopenia, delayed gametogenesis, and some behavioral abnormalities. In spite of these deficiencies, the availability of these mutant mice has allowed us to use them as tools to study the role of c-*fos* in cancer development in vivo. Our intention was to examine the unfolding of the neoplastic process in the absence of a critical component of the AP-1 family.

To address the relevance of c-*fos* for cancer development, we have used the classical model of multistep mouse skin carcinogenesis (reviewed by DiGiovanni, 1992; Hennings et al., 1993; Yuspa, 1994). Tumor devel-

opment in mouse skin can be divided into three steps: initiation, promotion, and progression. Initiation is an irreversible step that experimentally can be brought about by the application of a single dose of a mutagen (e.g., 2,5-dimethoxybenzaldehyde [DMBA]). Initiated cells can lie dormant until they are induced to proliferate, either by repeated treatments with a tumor promoter (e.g., 12-O-tetradecanoylphorbol-13-acetate [TPA]) or by a natural promoting stimulus such as wounding. During promotion, initiated cells are thought to have a growth advantage, and the tissue hyperplasia eventually results in a visible benign clonal outgrowth, a papilloma. Over time, papillomas can accumulate additional genetic mutations and progress into a malignant tumor, a squamous cell carcinoma. Carcinomas can undergo a further epithelial–mesenchymal transition to give rise to highly invasive spindle cell tumors (Klein-Szanto et al., 1989; Buchmann et al., 1991).

In this study, we have found that while c-*fos* does not seem to be necessary for normal epidermal differentiation or for the early proliferative steps of skin tumor formation, it is required for malignant tumor conversion. These results offer clear genetic evidence that an AP-1 factor is indispensable for the development of certain cancers.

Results

c-*fos*-Deficient Keratinocytes Form a Normal Epidermis

The skin is one of the few adult tissues in which c-*fos* expression has been reported to be constitutive (Fisher et al., 1991; Smeyne et al., 1992). Coupled with the observation that functional AP-1 sites can be found in a variety of genes associated with epidermal development, this finding raised the possibility that the skin of c-*fos* null mice could display an aberrant pattern of differentiation. To uncover any intrinsic abnormalities in unperturbed c-*fos*-deficient skin, we stained newborn epidermis with markers for specific stages of keratinocyte differentiation. Figure 1A shows double-labeled immunofluorescence staining for three markers: keratin 14 (K14), expressed in proliferating basal keratinocytes; keratin 1 (K1), induced in the differentiating spinous cells; and loricrin, a component of the cornified envelope expressed in the terminally differentiated cells of the granular layer. Comparison of the staining patterns for these and other epidermal differentiation markers failed to reveal any differences among mice of varying c-*fos* genotype. Histological examination of newborn c-*fos*-deficient skin showed that no obvious differ-

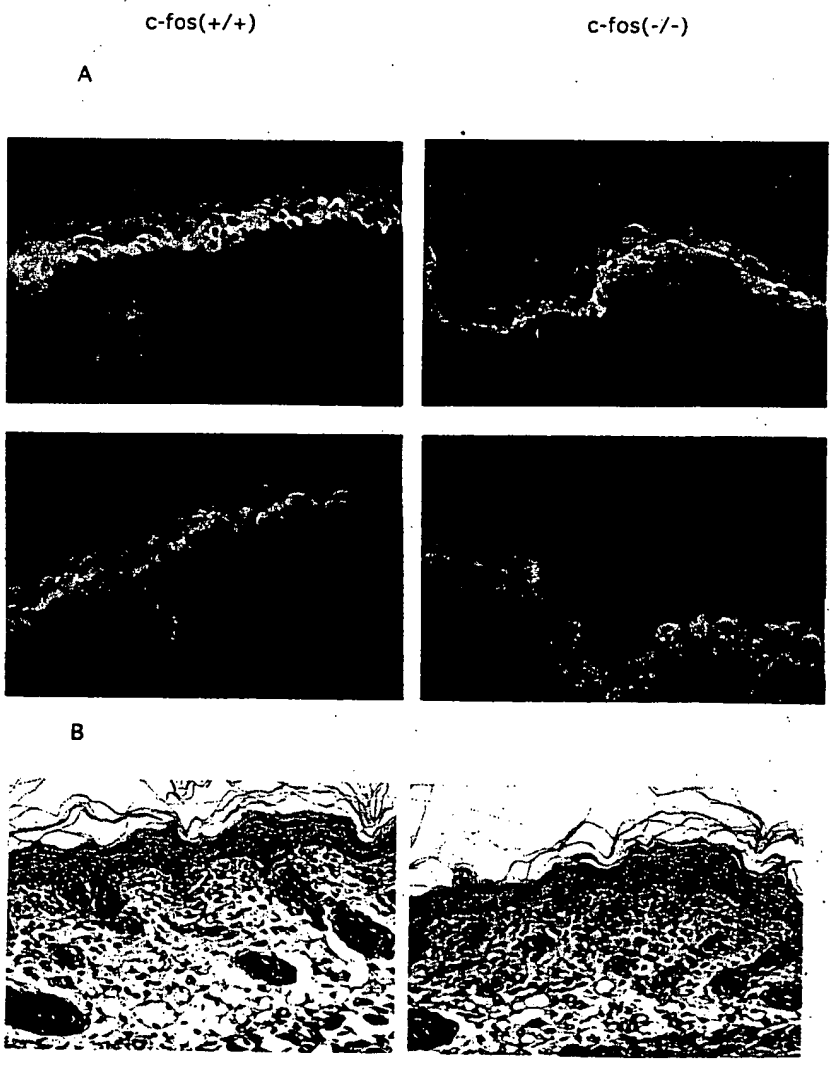

Figure 1. Analysis of Wild-Type and c-*fos*-Deficient Epidermis

(A) Indirect immunofluorescence staining of epidermal differentiation markers in newborn epidermis. In the top panels, K14 staining is shown in green and K1 in red. Areas of overlap appear yellow. The bottom panels show K14 in green and loricrin in red. Again, areas of common staining are yellow. Magnification, 204×.

(B) Hematoxylin and eosin staining of wild-type and c-*fos*-deficient newborn skin. Magnification, 163×.

ences exist in epidermal organization or cellularity (Figure 1B). Analogous results were obtained with thinner adult skin. Furthermore, when newborn mice were injected with bromodeoxyuridine to identify epidermal cells undergoing DNA replication, the number of proliferating cells was found to be similar in both genotypes (data not shown). These observations allowed us to conclude that, on the basis of keratinocyte growth rate or pattern of differentiation-dependent gene expression, the skin of c-*fos*-deficient mice is indistinguishable from that of wild-type mice.

c-*fos* Is Not Required for the Early Stages of Skin Carcinogenesis

To induce tumor formation in the skin of mice, we used a modified version of a traditional DMBA/TPA tumor induction protocol. Instead of using a chemical initiator, c-*fos* mutant mice were bred with a transgenic strain (TG.AC) carrying a v-H-*ras* transgene that can act as a classical initiation event (Leder et al., 1990). The existence of this transgenic strain enabled us to avoid the use of initiators that would have introduced undefined genetic mutations, potentially complicating the interpretation of the results. Even though the transgene is under the control of an embryonic globin promoter and thus is not normally active in the skin, expression of the transgene is induced in epidermal cells when they are prompted to proliferate either by application of tumor promoters or by a natural stimulus such as hair plucking (Hansen and Tennant, 1994a, 1994b). Mice carrying the TG.AC transgene are very sensitive to a variety of tumor promoters; they rapidly develop large numbers of papillomas (Spalding et al., 1993).

Mice homozygous for the TG.AC transgene were mated with mice heterozygous for the c-*fos* null mutation. Offspring carrying both mutations were identified and interbred to generate mice of all possible genotypes. The dorsal epidermis of these mice was then treated with the tumor promoter TPA twice a week for 5 weeks, according to standard promotion protocols for the TG.AC strain. Treated mice were monitored biweekly for the appearance of papillomas. As can be discerned from Figure 2, c-*fos*-deficient mice were able to develop papillomas, and they did so with equivalent kinetics to those of their wild-type and heterozygous littermates. Furthermore, the time to appearance of the first tumor and the number of papillomas per mouse were indistinguishable among genotypes. Because papillomas are very heterogenous and often tend to coalesce with other papillomas, size comparisons are difficult. Nevertheless, no obvious differences in tumor size could be observed among mice of differing genotypes.

Table 1 summarizes benign tumor incidence rates. A small minority of v-H-*ras* transgenic mice in each group proved to be resistant to TPA treatment. This resistance did not correlate with c-*fos* genotype and has previously been described for inbred FvB/N TG.AC mice. Two conclusions can be drawn from the data in Table 1 regarding the role of c-*fos* in the early steps of mouse skin carcinogenesis. First, the absence of c-*fos* cannot act as an initiat-

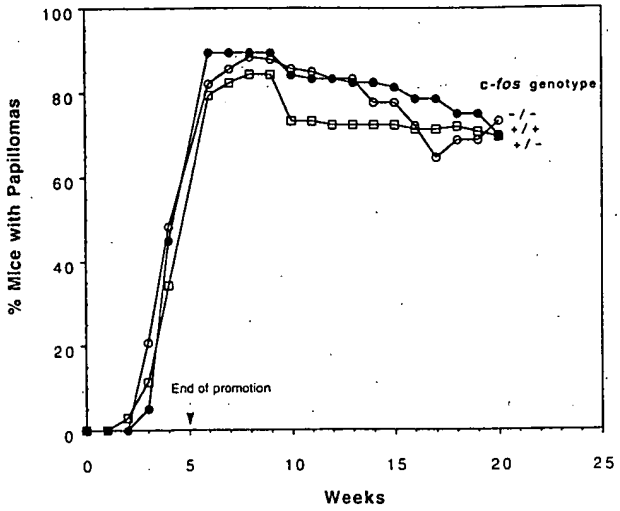

Figure 2. Initial Kinetics of Papilloma Appearance

Number of papilloma-bearing mice as a percentage of those treated with tumor promoter: wild-type mice (closed circles; n = 19), heterozygous mice (open squares; n = 35), and c-*fos* null mice (open circles; n = 29).

Second, since c-*fos*-deficient mice bearing the v-H-*ras* transgene developed papillomas in a normal manner, it is clear that in this model of carcinogenesis, this proto-oncogene is not necessary for promotion.

c-*fos*-Deficient Papillomas Evolve into Horny, Elongated Tumors Characterized by Massive Hyperkeratinization

Although c-*fos*-deficient papillomas initially displayed an identical external appearance to wild-type tumors, a striking change in morphology was noticed soon after the end of promotion. Within 4 weeks after TPA treatment was completed, the papillomas on all c-*fos* null mice started to become very dry, elongated, and hyperkeratinized. This change in morphology was very apparent as the study advanced. Though some papilloma regression was observed in mice of all genotypes, the papillomas that persisted on wild-type and heterozygous mice remained vigorous and well vascularized. In contrast, mutant papillomas became desiccated and extremely hyperkeratinized and showed little external vascularization. Figure 3A shows a comparison of wild-type and c-*fos* null papillomas soon after the end of promotion. While wild-type papillomas retained a similar morphology until the end of the study, c-*fos*-deficient tumors acquired a rather horny appearance: they evolved into severely keratinized, grotesque projections. Examples of these older c-*fos*-deficient tumors are shown in Figure 3C. Histopathology of wild-type and mutant papillomas revealed that c-*fos*-deficient tumors indeed display a very dramatic hyperkeratinization, showing a remarkable increase in the terminally differentiated stratum corneum (Figure 4). In addition, c-*fos*-deficient papillomas seemed to present an abrupt transition between the basal layer and the stratum corneum,

Table 1. Benign Tumor Incidence Rates

Genotype	Treatment	Papillomas	Incidence (%)	Papillomas per Mouse	Time to First Tumor
c-*fos* wild-type transgenic v-H-*ras*	TPA	17 of 19	90	26 ± 14.4	33.4 ± 7.2
	Acetone	0 of 4	0		
c-*fos* heterozygous transgenic v-H-*ras*	TPA	30 of 35	86	22 ± 15.7	34.8 ± 8.9
	Acetone	0 of 5	0		
c-*fos*-deficient transgenic v-H-*ras*	TPA	26 of 29	90	23 ± 14.3	32.0 ± 7.7
	Acetone	0 of 6	0		
c-*fos* wild type	TPA	0 of 9	0		
c-*fos* heterozygous	TPA	0 of 13	0		
c-*fos* deficient	TPA	0 of 6	0		

At 12 weeks of age, all mice were shaved and promotion treatment was started either with TPA or with solvent alone (acetone). Mice were monitored biweekly for the appearance of tumors. Papillomas per mouse and time to first tumor statistics are given as mean ± SD.

the c-*fos* null mutation appeared identical to wild-type papillomas: their morphology did not change, and they did not present an abnormal epidermal architecture.

To investigate whether these hyperkeratinized c-*fos*-deficient tumors showed an abnormal rate of regression, we compared the average number of papillomas per mouse at 9 weeks and at 23 weeks. Since TPA treatment was discontinued at the end of week 5, the majority of regressing papillomas should have disappeared by week 23. In wild-type mice, the mean number of papillomas per mouse decreased from 26 at week 9 to 10.5 at week 23 (a 60% reduction). Heterozygous mice showed a very similar decline, from 22.1 tumors per mouse at week 9 to 8.9 at week 23 (a 60% decrease). The mean number of papillomas per mouse in c-*fos*-deficient animals decreased from 23 at week 9 to 4.7 at week 23 (an 80% reduction). These data show that c-*fos* is not required for the persistence of papillomas. Whether papilloma regression rates are significantly affected by the c-*fos* null mutation is an issue that will require more study.

Papillomas Lacking c-*fos* Fail to Undergo Malignant Conversion

Wild-type mice began to develop malignant skin tumors around week 20, although most cases of malignant progression were noticed between weeks 25 and 30. Malignant lesions first appeared as ulcerated areas within a papilloma that grew rapidly. These malignant tumors were identified histologically as either squamous cell carcinomas or, more frequently, spindle cell tumors, in agreement with what has been described for the TG.AC strain (French et al., 1994). By the end of the study (week 45), malignant progression had been observed in 4 of 7 (57%) surviving wild-type mice and in 7 of 17 (41%) heterozygous mice (Table 2). In contrast, no malignant skin tumors had been detected in 13 remaining c-*fos* null mice. A detailed pathological examination of all remaining c-*fos*-deficient papillomas at the end of the study failed to find any evidence of microscopic malignant progression. The decrease in the number of mice that were available at the later timepoint to assess progression is a reflection of the fact that the v-H-*ras* TG.AC transgene induces a variety of other tumors (e.g., hematopoietic malignancies) that necessitate the sacrifice of the affected animal. Nevertheless, the difference in progression rates of tumors with or without c-*fos* (57% and 41% versus 0%) is statistically significant ($p < 0.0001$) and allows us to conclude that c-*fos* is required for malignant progression of experimentally induced skin tumors. Wild-type and c-*fos*-deficient papillomas expressed similar levels of transgenic v-H-*ras* 1 month after the end of tumor promoter treatment (Figure 5B). Hence, the inability of c-*fos*-deficient papillomas to become malignant is not due to a reduced level of oncogenic H-*ras* expression. These final progression figures do not exclude the possibility of a heterozygous effect, although the finding that heterozygous papillomas behave like wild-type papillomas by all criteria examined argues against it.

Alterations in Gene Expression in c-*fos* Null Papillomas

To examine whether the lack of malignant progression of c-*fos*-deficient tumors correlated with specific changes in gene expression, we isolated RNA from pools of papillomas from wild-type and c-*fos*-deficient mice. Each pool consisted of five to six tumors taken 1 month after the end of TPA treatment. These RNAs were used to measure the degree of expression of certain AP-1-regulated genes in the papillomas. The genes encoding the tumor metalloproteases stromelysin and type I collagenase were of particular interest because studies using c-*fos*-deficient fibroblasts had shown that c-*fos* is required for the induction of these genes in response to mitogenic stimulation (Hu et al., 1994). In addition, enhanced expression of these tumor metalloproteases has been associated with the progression of benign, encapsulated papillomas to malignant, invasive tumors (Matrisian et al., 1986; Liotta and Stetler-Stevenson, 1990). While the stromelysin and type I collagenase mRNAs were detectable in the pools of wild-type tumors, they were virtually absent in RNA pools from c-*fos*-deficient papillomas (Figures 5A and 5B). The apparent lack of external vascularity of mutant tumors also prompted us to assess the level of expression of angiogenic factors in the papillomas. Of special interest was the pattern of expression of vascular endothelial growth

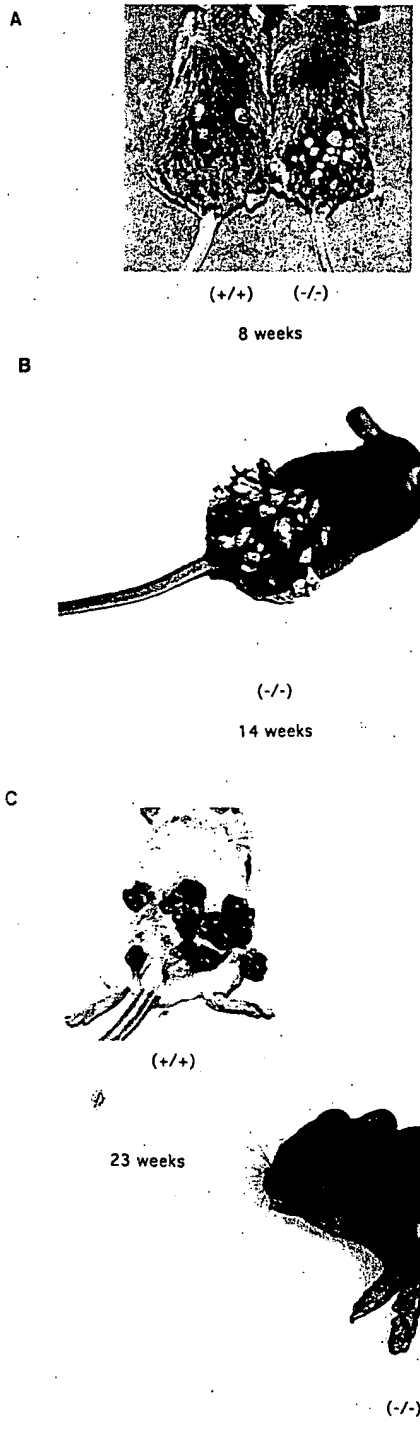

Figure 3. External Appearance of Papillomas

(A) A wild-type mouse (left) and a c-*fos*-deficient littermate (right) are shown 3 weeks after the end of promotion.

(B) c-*fos* null mouse 9 weeks after the end of promotion (14 weeks from the start of treatment); note the change in morphology that is now obvious in all papillomas.

(C) Wild-type and c-*fos*-deficient tumors 4 months after the conclusion of TPA treatment. The black mouse is the same mouse as that shown in (B). Notice the absence of external vascularity in the large tumors of the brown c-*fos*-deficient mouse. Color coat segregates independently of both the TG.AC transgene and the c-*fos* null mutation.

factor (VEGF), for this protein may be the prime regulator of normal and tumor angiogenesis (Klagsbrun and Soker, 1993). The VEGF gene was expressed in c-*fos*-deficient tumors, but the levels of VEGF mRNA measured in mutant papillomas were 5- to 10-fold lower than those found in RNA pools derived from wild-type papillomas (Figure 5C).

The changes in keratin gene expression that take place during a protocol of skin carcinogenesis are well established and can often serve as markers of the degree of tumor progression. For example, papillomas with a high risk for malignant conversion frequently replace K1 expression with that of K13, a keratin whose expression is normally restricted to internal epithelia (Nischt et al., 1988; Gimenez-Conti et al., 1990). Regions of premalignant papillomas that express K13 do not normally express K1. To monitor keratin gene expression in c-*fos*-deficient papillomas, we stained serial sections of tumors with antibodies prepared against an array of keratins. Surprisingly, K13 expression was ubiquitous in the suprabasal layers of c-*fos*-deficient papillomas. More significantly, though, while K1 and K13 expression was mutually exclusive in wild-type papillomas, these two keratins were invariably coexpressed in the same cells in the mutant papillomas. K1 expression persisted in spite of K13 induction in all

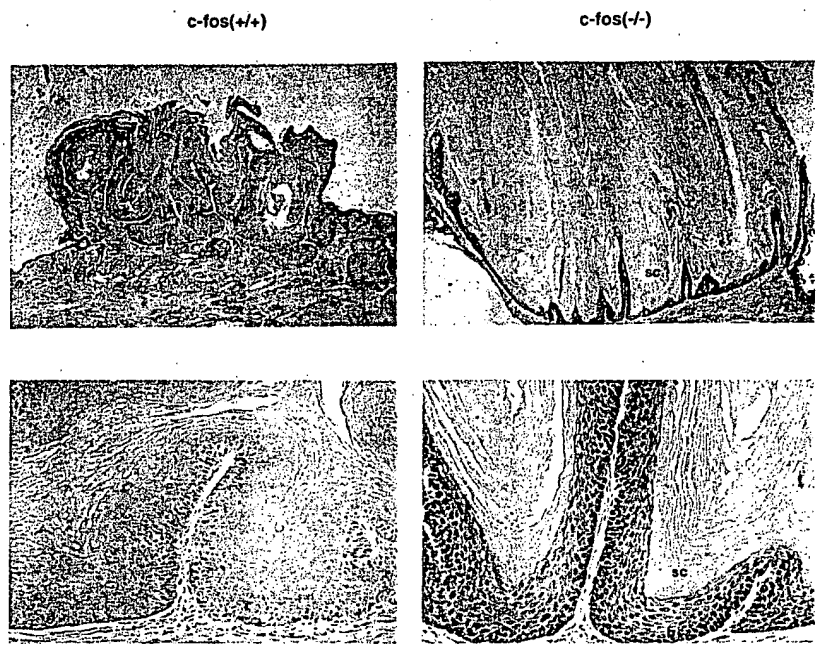

Figure 4. Histology of Papillomas

Hematoxylin and eosin–stained sections of 2-month-old tumors (3 weeks after TPA removal). The top panels show a wild-type and a c-*fos*-deficient tumor at the same level of magnification (13 ×). Note the striking hyperkeratinization of the *fos*-less papilloma and the abrupt transition between the basal (b) and the cornified layers (sc). At higher magnification (135 ×; same for both genotypes), the contraction of the layers in the c-*fos*-deficient tumor becomes more evident.

c-*fos*-deficient papillomas examined (Figure 6). Since these type 1 and type 2 keratins do not normally form filament pairs, coexpression of K1 and K13 in the same cells could result in the formation of an irregular intermediate filament network that may contribute to the unusual amount of cornification displayed by mutant papillomas. In contrast with wild-type cornified envelopes, which disassociate when tumors are boiled, large clumps of tightly connected c-*fos*-deficient envelopes endured even after the mutant papillomas were boiled for 5 hr in 2% SDS (data not shown). The considerable durability of c-*fos*-deficient cornified envelopes may reflect interesting underlying biochemical differences, such as covalent modifications in the structure of the envelopes.

c-*fos*-Deficient Keratinocytes Expressing v-H-*ras* Do Not Form Tumors When Grafted onto Nude Mice

The development of a malignant neoplasm is a process that involves an intense interaction between tumor cells and normal neighboring cells (reviewed by Paweletz and Boxberger, 1994). The inability of c-*fos*-deficient papillomas to progress into malignant tumors could be due to an intrinsic defect in the v-H-*ras*-expressing keratinocytes that form the epidermal component of the papillomas. Alternatively, it is possible that malignant tumors do not arise in these animals because the nontransformed c-*fos*-deficient cells neighboring the neoplastic keratinocytes do not provide an environment that is conducive for malignant

Table 2. Incidence of Malignant Tumors

Genotype	Malignant Tumors	Percent Affected
c-*fos* wild-type transgenic v-H-*ras*	4 of 7	57
c-*fos* heterozygous transgenic v-H-*ras*	7 of 17	41
c-*fos* deficient transgenic v-H-*ras*	0 of 13	0

Mice were monitored weekly during 45 weeks for the appearance of malignancies. At the end of that time, all mice were sacrificed, and any remaining tumors were harvested for pathological analysis. Individual mice were sacrificed after the first malignant tumor was detected.

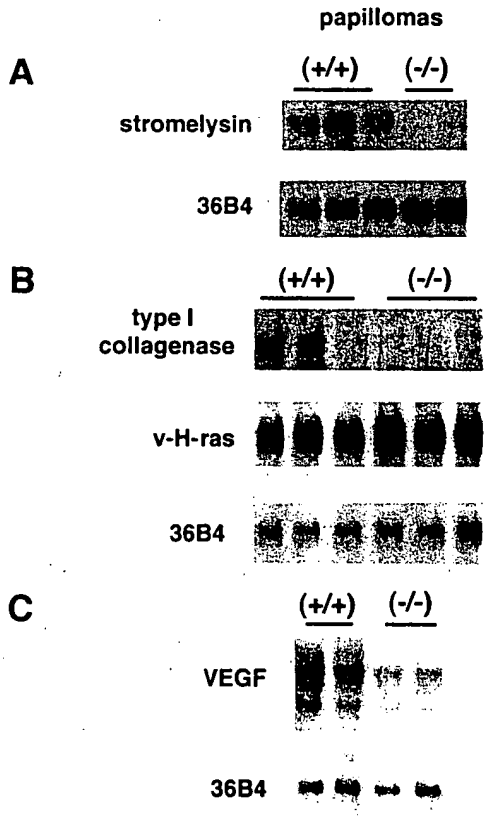

Figure 5. Expression of c-*fos*-Regulated Genes in Papillomas

RNA was prepared from papillomas 1 month after the end of promotion and analyzed by Northern blot. Each lane contains total RNA from a pool of six to eight tumors. Probes are as indicated on each panel. Blots were stripped and reprobed with ribosomal-associated protein 36B4 as a control for loading.

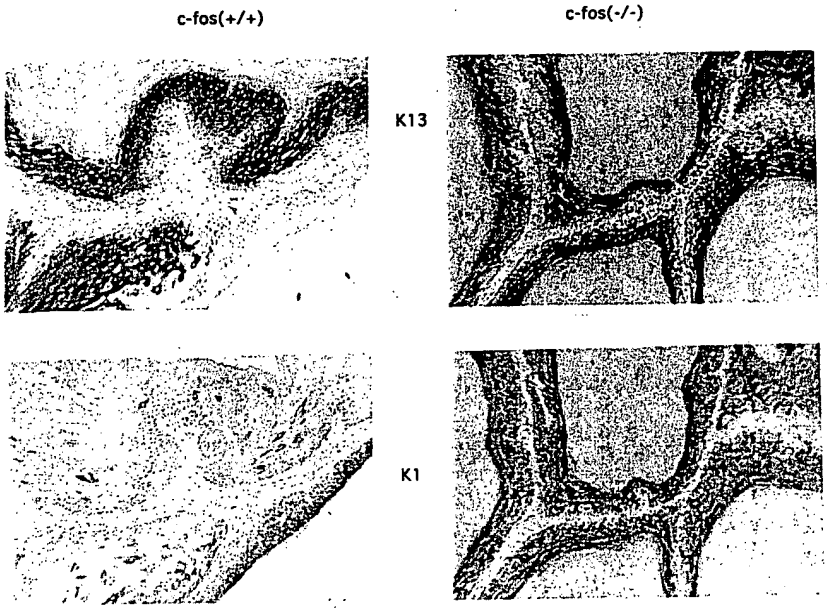

Figure 6. Aberrant Keratin Gene Expression in c-*fos*-Deficient Papillomas

Serial sections of tumors (obtained 2.5 months after the start of the study) were stained with antibodies against K1 (bottom) and K13 (top) and photographed at the same magnification (32×). Note the coexpression of K1 and K13 in c-*fos*-deficient tumors.

growth. To establish whether c-*fos*-deficient keratinocytes display a cell-autonomous defect that prevents them from giving rise to malignant tumors, we tested the ability of v-H-*ras*-expressing mutant keratinocytes to form tumors when grafted onto the back of wild-type hosts (Roop et al., 1986). Primary keratinocytes derived from c-*fos*-deficient, heterozygous, and wild-type mice were infected with a helper-free retrovirus, a variant Harvey murine sarcoma virus (Ha-MSV), expressing an oncogenic form of H-*ras* (Roop et al., 1986), were mixed with wild-type dermal fibroblasts, and were grafted onto the back of athymic nude mice. Grafted Ha-MSV-infected wild-type keratinocytes gave rise to large papillomas. In contrast, grafts of infected c-*fos*-deficient keratinocytes did not produce significant tumors. Rather, v-H-*ras*-expressing cells lacking c-*fos* generated a normal epidermis that was hyperplastic in some cases. Interestingly, grafts of keratinocytes heterozygous for the c-*fos* null mutation gave rise to small tumors of variable size that on average were approximately 35% the size of their wild-type counterparts. While there was some variation in absolute tumor volume values, the same trend was observed in three independent experiments: v-H-*ras*-expressing keratinocytes homozygous for the c-*fos* null mutation failed to produce tumors (n = 8), heterozygous cells generated small, fragmented papillomas (n = 12), and wild-type keratinocytes gave rise to large tumors (n = 14). Mice from a representative experiment are shown in Figure 7A. The presence of black pigment in mice with grafts of c-*fos*-deficient keratinocytes indicates that the graft was successful. Figure 7B displays the evolution of tumor volume as a function of time for that same experiment. Mice with c-*fos*-deficient grafts failed to develop tumors, even when monitored for 3 months (6 weeks beyond the normal observation period). Since tumor formation in this system is dependent upon oncogenic H-*ras* expression, the levels of mutant Ras protein in keratinocytes used for grafting were examined. All Ha-MSV-equivalent levels of exogenous Ras in vitro as well as in vivo (data not shown). The growth rate in vitro of all v-H-*ras*-expressing keratinocytes was similar, regardless of c-*fos* genotype (data not shown). Furthermore, implanted c-*fos* null keratinocytes coinfected with Ha-MSV and a v-*fos* retrovirus generated dysplastic tumors indistinguishable from those produced by similarly infected wild-type cells, confirming that a *fos*-dependent function is responsible for the deficiency in tumor formation of mutant cells (data not shown). These transplantation experiments illustrate that v-H-*ras*-expressing keratinocytes derived from c-*fos* null animals appear to have a cell-autonomous defect that hampers tumorigenesis.

Discussion

Tumorigenesis is a complex process that requires alterations in many genes involved in the regulation of cell growth. Many of these genes function outside of the nucleus of the cell and must ultimately exert their effects by communicating with transcription factors. The AP-1 family is believed to be one of the nuclear targets of several signal transduction pathways, in particular the Ras–MAP kinase pathway (reviewed by Hill and Treisman, 1995). Because these pathways are frequently dysregulated by activated oncogenes, it is of considerable interest to determine whether AP-1 factors are actually necessary for cancer development. We have used the well-characterized model of multistep mouse skin carcinogenesis to examine the unfolding of the neoplastic process in the absence of c-*fos*, an important member of the AP-1 family. Our results demonstrate that c-*fos* is required for the development of malignant skin tumors.

The Role of c-*fos* in Initiation and in Promotion

To test the role of c-*fos* in the various steps of skin carcinogenesis, we mated mice heterozygous for the c-*fos* null mutation with a transgenic strain carrying a v-H-*ras* transgene

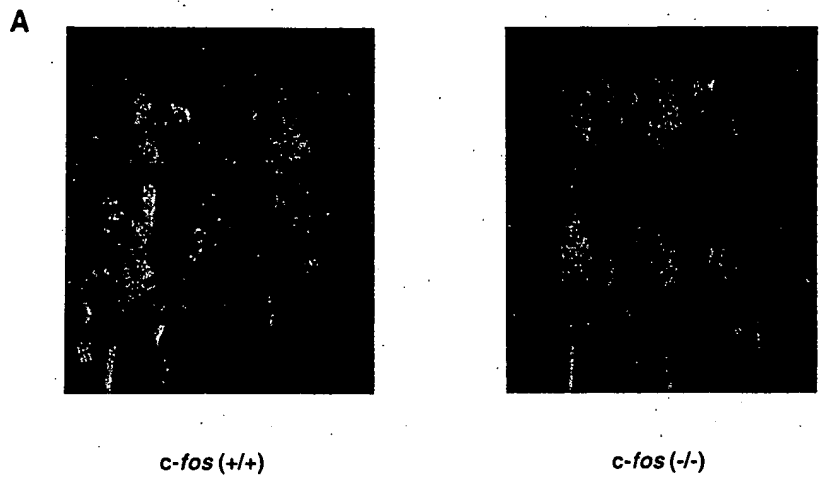

Figure 7. Grafting of v-H-*ras*-Expressing Keratinocytes

(A) Appearance of grafts of Ha-MSV-infected keratinocytes 6 weeks after implantation. The genotype of grafted keratinocytes is shown below each picture. The presence of pigment (denoting cells of the C57BL/6J × 129/SvJ genetic background) indicates that grafts were successful.

(B) Tumor development as a function of time for grafts of wild-type (n = 4), heterozygous (n = 5), and c-*fos*-deficient (n = 3) v-H-*ras*-expressing keratinocytes. Curves represent the average tumor volume for each group. Error bars denote SEM. Note that c-*fos*-deficient cells did not give rise to tumors.

that can substitute for a chemical initiation step. Even though mutations in H-*ras* are very common during initiation in DMBA/TPA protocols, skin tumors induced with other carcinogens frequently do not bear H-*ras* mutations (Bremner et al., 1994). This observation suggests that mutations in other unspecified genes can also serve as initiating events. To examine whether c-*fos* could be one of these unidentified genes, we treated c-*fos* null mice not carrying the v-H-*ras* transgene with the tumor promoter TPA. The absence of papilloma formation established that a c-*fos* null mutation cannot serve as an initiation event.

Mutant mice bearing the v-H-*ras* transgene were able to develop papillomas normally upon TPA treatment. That c-*fos*-deficient papillomas appeared with standard kinetics and in comparable numbers to wild-type tumors shows that, under these experimental conditions, c-*fos* is not required for benign tumor formation. Because c-*fos* is an immediate-early gene whose expression is often used as a marker of cellular proliferation, it may have been anticipated that the main contribution of c-*fos* to oncogenesis would be either to accelerate the rate of cell division or to prevent exit from the cell cycle. Clearly, this does not seem to be the case in this study. It is possible that during promotion, TPA induces the expression of other genes that can functionally complement the absence of c-*fos*. Nonetheless, since in the vast majority of experimental carcinogenesis protocols, TPA promotion is continued for many weeks, the real relevance, if any, of c-*fos* for tumor promotion would also be concealed under those circumstances. Thus, we can conclude that, while it may be important, c-*fos* is not essential for tumor promotion brought about by TPA treatment.

Abnormalities of Epidermal Differentiation in c-*fos*-Deficient Papillomas

Although initially identical to wild-type papillomas, shortly after the end of TPA treatment, c-*fos*-deficient tumors began to manifest some clear differences. Over time, c-*fos*-deficient papillomas are characterized by a grotesque hyperkeratinization. Mutant papillomas evolve into horny projections covered by a crust of keratin that can grow as long as 2–3 cm. The development of such elongated, hyperkeratinized papillomas (with the great frequency found here) seems to be unique for a carcinogenesis proto-

col. It was surprising to find universal K13 expression in these apparently benign tumors, for K13 induction has heretofore been considered a useful diagnostic of premalignant progression. It is important to note that these c-*fos*-mediated differences in epidermal differentiation are only apparent within the context of carcinogenesis, for no differentiation abnormalities were detected in untreated c-*fos*-deficient epidermis. Hence, it is conceivable that the observed differences are contingent on the expression of a mutant H-Ras protein. It is also possible that the expression of the various members of the AP-1 family may be abnormally regulated during tumorigenesis. Given the large number of AP-1 sites that have been identified in epidermal differentiation genes, disorderly expression of other AP-1 members in the absence of c-*fos* may have profound effects. Using immunohistochemistry, we have been unable to recognize significant differences in the expression pattern of AP-1 genes in wild-type and mutant papillomas (data not shown). However, since the activity of AP-1 proteins can be regulated by reversible phosphorylation, it is possible that differences may exist in AP-1-dependent gene expression that are not reflected in an unusual pattern of AP-1 family protein expression.

The Role of c-*fos* in Papilloma Survival and Malignant Progression

In contrast with wild-type and heterozygous tumors, not a single c-*fos*-deficient papilloma progressed to malignancy by the end of the study. Several explanations can potentially account for this observation. It is well established that c-*fos* controls the expression of a variety of genes that are up-regulated during malignant progression, including those encoding the tumor metalloproteases stromelysin and type I collagenase (Kerr et al., 1988; Schonthal et al., 1988; Hennigan et al., 1994). The tumor metalloproteases are a group of secreted enzymes that can degrade the extracellular matrix, thereby facilitating tumor growth, invasion, and metastasis (reviewed by Liotta and Stetler-Stevenson, 1990). Increased metalloprotease expression has been associated with malignant progression in many models of in vivo cancer development (reviewed by McDonnell and Matrisian, 1990). Given that the transcripts for stromelysin and type I collagenase were undetectable in RNA obtained from pools of mutant papillomas, it is likely that a protease insufficiency may contribute to the lack of malignant behavior of c-*fos*-deficient tumors.

The sparse external vascularity displayed by c-*fos*-deficient papillomas suggests a second possible factor for the benign nature of c-*fos*-deficient tumors. At a molecular level, the formation of new blood vessels is a process remarkably similar to tumor invasion and metastasis, for angiogenesis also requires cells to traverse normal tissue boundaries and degrade the basement membrane of the tissue to be vascularized (reviewed by Liotta et al., 1991). c-*fos* could be mediating tumor angiogenesis not only through its control of the proteases that are critical for these processes, but also by modulating the expression of the growth factors that induce neovascularization, such as VEGF. Indeed, we have observed that the level of VEGF pools of c-*fos*-deficient tumors. Finally, it is conceivable that the abnormal pattern of differentiation of c-*fos*-deficient papillomas may interfere or be incompatible with ordinary tumor progression.

Diminished expression of tumor metalloproteases and angiogenic factors may also contribute to the inability of grafted v-H-*ras*-expressing c-*fos*-deficient keratinocytes to generate papillomas. The development of an established graft/tumor involves complex fibroblast–keratinocyte interactions that must ultimately result in, among other things, the creation of a new blood vessel network to support the growth of the graft (Smola et al., 1993; Borchers et al., 1994). In the multistep carcinogenesis system, the requirement for angiogenesis may be less stringent, at least for the early stages of tumor development. Since tumors are induced in skin that is already vascularized, initially neoplastic cells need only to expand the existing capillary network, not to establish a completely new one. It is also possible that chemical treatment in the multistep carcinogenesis system induces the expression of genes that allow papilloma formation in the absence of c-*fos*. Many genes, including those encoding the tumor metalloproteases stromelysin and type I collagenase, are known to be expressed in response to tumor promoter stimulation; c-*fos*-deficient fibroblasts can express a limited amount of these proteases when stimulated with TPA (Krieg et al., 1988; Hu et al., 1994). The defect in tumor development observed in the grafting system could also be due to an autocrine overproduction of a growth-inhibiting molecule (e.g., transforming growth factor β) by c-*fos*-deficient cells. Alternatively, v-H-*ras*-expressing mutant cells may be more sensitive to constraining signals emanating from the wild-type cells that constitute the stromal component of the implant. The experimental flexibility of the skin grafting system should permit the reintroduction of proteases and angiogenic factors to test whether the cell-autonomous defect in tumorigenesis of c-*fos*-deficient keratinocytes is linked to the expression of these genes.

Although the specific details of why c-*fos*-deficient papillomas fail to progress to malignancy are yet to be elucidated, these findings might provide a molecular mechanism for the observation that treatment with retinoids and glucocorticoids can prevent malignant progression of mouse skin tumors, as well as the occurrence of secondary tumors in carcinomas of the head and neck in humans (Hong et al., 1990; Strawhecker and Pelling, 1992; De Luca et al., 1993). Both, the retinoic acid receptors and the glucocorticoid receptor are known to antagonize AP-1-dependent transcription directly (Schule et al., 1990; Kerppola et al., 1993). Thus, it is possible that the inhibitory action of these hormones on AP-1 activity is at least partially responsible for their antitumorigenic effects.

Relevance to Other Neoplastic Processes

An important question is whether these findings will be meaningful for tissues other than the epidermis. We have indirectly addressed this issue by monitoring the frequency of the spontaneous tumors induced by the v-H-*ras* TG.AC transgene in c-*fos*-deficient mice. We have found

nonsolid neoplasms, it appears to be critical for the development of certain solid tumors, such as odontogenic fibrosarcomas (unpublished data). Moreover, a variety of other human and rodent tumors are known to overexpress c-*fos* (e.g., Honoki et al., 1992; Urabe et al., 1992). One of these tumors is human squamous cell carcinoma of the lung (Volm et al., 1992; Wodrich and Volm, 1993). Because the development of this cancer naturally requires repeated applications of a tumor promoter (e.g., tobacco smoke), many consider this human cancer the most accurate reflection of the mouse skin model. Another clinically relevant tumor in which c-*fos* overexpression is associated with malignant progression is human breast cancer (Biunno et al., 1988; Walker and Cowl, 1991).

Our findings highlight the significance of c-*fos* for full neoplastic development. Since malignant progression is the most critical step in terms of host survival, the identification of a gene central to this process represents a promising step toward the therapeutic prevention of neoplastic disease. Furthermore, because c-*fos* is not required for the viability of the whole organism, it may represent an attractive target for pharmacological intervention.

Experimental Procedures

Animals

c-*fos*-deficient mice were generated as previously described (Johnson et al., 1992). Mice heterozygous for the c-*fos* null mutation (50% 129/SvJ; 50% C57BL/6J) were mated with FvB/N mice homozygous for the TG.AC transgene obtained from Charles River Laboratories. Offspring carrying both the TG.AC transgene and the c-*fos* mutation were identified and interbred. Their progeny was typed for both mutations and divided into the groups shown in Table 1. At 12 weeks of age, mice were shaved, and their dorsal epidermis was treated with either 5 μg of TPA (Sigma) dissolved in 100 μl of acetone or acetone alone. To normalize for their reduced size (and hence smaller dorsal surface area), c-*fos*-deficient mice received 55% of the wild-type dose of promoter. This dose of TPA resulted in a similar papilloma yield in wild-type and mutant mice (see Table 1). Treatment was applied twice a week for 5 weeks. Male mice were caged individually to prevent wound-induced tumors. All mice were examined at least once a week for a period of 45 weeks.

Histology and Immunohistochemistry

For pathological analysis, complete autopsies were performed and tissues were fixed in Optifix. Tumors were not removed from mice under observation. For fluorescent immunohistochemistry, neonatal skin was frozen in OCT (Miles Incorporated). Double-labeled indirect immunofluorescence was performed as described by Nischt et al. (1988). Antisera against mouse keratins and loricrin are characterized by Yuspa et al. (1989) and Mehrel et al. (1990).

Cell Culture and Retroviral Infections

Breeding pairs of animals heterozygous for the c-*fos* null mutation were monitored daily for the appearance of offspring. Neonatal mice were genotyped by PCR (Johnson et al., 1992). After sacrifice, animals were kept on ice until typing results became available (24 hr). The skin from selected mice was removed, and primary keratinocytes were prepared as described by Hennings et al. (1980). Dermal fibroblasts from the same c-*fos* wild-type mice used to derive keratinocytes to be grafted were cultured separately for 1 week (Yuspa et al., 1976). Primary keratinocytes were infected 3 days after plating at a multiplicity of 1 with a replication-defective variant of Ha-MSV devoid of helper virus and containing the v-H-*ras* gene (Roop et al., 1986). After 5 days, keratinocytes and fibroblasts were combined and grafted. Wild-type fibroblasts were used for grafting keratinocytes of all three genotypes. Successful Ha-MSV infection and p21 H-Ras expression were monitored by analyzing total protein extracts of infected keratinocytes by Western blot and by immunohistochemistry on grafts, using an antibody against p21 H-Ras (Transduction Laboratories).

In Vivo Grafting

Combined cell pellets were transplanted onto 8- to 12-week-old athymic nude mice as described by Strickland et al. (1993). The volume of tumors was measured on a weekly basis, starting 2 weeks after grafting. Mice were sacrificed after 6 weeks of observation, unless otherwise noted.

Statistical Analysis

Statistical work was performed by the Division of Biostatistics at the Dana–Farber Cancer Institute. The associations between c-*fos* genotype and the incidence of epidermal tumors were analyzed with log linear models using GLIM (GLIM4, 1992 Royal Statistical Society, London), evaluating the association both assuming and not assuming and ordering to the genotype (+/+ > +/− > −/−); p values of <0.05 were considered significant.

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c-*fos*-induced growth factor/vascular endothelial growth factor D induces angiogenesis *in vivo* and *in vitro*

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ABSTRACT **c-*fos*-induced growth factor/vascular endothelial growth factor D (Figf/Vegf-D) is a secreted factor of the VEGF family that binds to the vessel and lymphatic receptors VEGFR-2 and VEGFR-3. Here we report that Figf/Vegf-D is a potent angiogenic factor in rabbit cornea *in vivo* in a dose-dependent manner. *In vitro* Figf/Vegf-D induces tyrosine phosphorylation of VEGFR-2 and VEGFR-3 in primary human umbilical cord vein endothelial cells (HUVECs) and in an immortal cell line derived from Kaposi's sarcoma lesion (KS-IMM). The treatment of HUVECs with Figf/Vegf-D induces dose-dependent cell growth. Figf/VEGF-D also induces HUVEC elongation and branching to form an extensive network of capillary-like cords in three-dimensional matrix. In KS-IMM cells Figf/Vegf-D treatment results in dose-dependent mitogenic and motogenic activities. Taken together with the previous observations that *Figf/Vegf-D* expression is under the control of the nuclear oncogene c-*fos*, our data uncover a link between a nuclear oncogene and angiogenesis, suggesting that Figf/Vegf-D may play a critical role in tumor cell growth and invasion.**

During development and in the vascularization of tumors inductive signaling leads to the formation of capillaries throughout the new-forming tissues (1, 2). Inhibitors who regulate proliferation, migration, differentiation of endothelial cells, degradation of the extracellular matrix, and tube formation finely tune this complex process, known as angiogenesis (3–5). The prototype of angiogenic factors is represented by vascular endothelial growth factor (VEGF) A, also known as vascular permeability factor. VEGF-A induces endothelial cell differentiation and is essential for embryonic vessel development (4, 6, 7). It belongs to a multigene family of angiogenic factors in which several new members were discovered recently. It generally is thought that each member of this family plays a specific role in the angiogenic process (2). In addition to VEGF-A, this family includes the placental growth factor (PlGF), VEGF-B/VRF, VEGF-C/VRP, c-*fos*-induced growth factor (Figf)/VEGF-D, and VEGF-E (8–18). All of these factors show a conserved cysteine-rich domain characteristic of the family. Differences in the patterns of expression suggest a specific role for at least some of the factors in the vascularization of different tissues. Plgf is expressed mostly in the placenta, whereas VEGF-B is prevalent in skeletal and cardiac muscle tissues (12, 19). These two factors can form heterodimers with VEGF-A adding an additional level of specificity (12, 20). Interestingly, VEGF-C is involved in both blood and lymphatic vessel growth (21, 22).

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Figf/Vegf-D initially was identified by using a differential screening strategy aimed at the identification of new c-*fos*-responsive genes in mouse fibroblasts and therefore named c-*fos*-induced growth factor (15). Its human orthologue shares 84% identity and was named *VEGF-D* because it encodes for a secreted protein whose primary sequence is most similar to VEGF-C (16, 23–25). Both VEGF-C and VEGF-D are recognized by VEGF receptors (VEGFR)-2 and -3, which are present on endothelial cells (14, 23). In mouse embryos *Figf/Vegf-D* is expressed in several organs, including limb buds, teeth, heart, and pituitary as well as lung and kidney mesenchyme, liver, derma, and periosteum of the vertebral column that partially overlaps *Vegf-C* expression (26, 27). In cultured fibroblasts *Figf/Vegf-D* regulation differs from *VEGF-C*. Whereas the expression of *Figf/Vegf-D* depends on c-*fos* (15), VEGF-C is induced by serum, tumor promoter phorbol myristate 13-acetate, IL-1β, and tumor necrosis factor α, and its expression is independent from c-*fos* (15, 28, 29).

We produced a recombinant form of mature mouse Figf/Vegf-D and analyzed its biological activity both *in vivo* and *in vitro*. Figf/Vegf-D, expressed in Chinese hamster ovary (CHO) cells or purified from yeast, is a potent angiogenic factor in rabbit cornea assays. *In vitro* it activates tyrosine phosphorylation of VEGFR-2 and VEGFR-3 present on human umbilical cord vein endothelial cells (HUVECs) and on the Kaposi's sarcoma immortalized cell line (KS-IMM). In KS-IMM cells Figf/Vegf-D induces proliferation and chemotaxis. In HUVECs Figf/Vegf-D induces growth and morphological changes within a three-dimensional matrix.

MATERIALS AND METHODS

Expression of Figf/Vegf-D. To express the mature factor in CHO cells, the Figf/Vegf-D cDNA with a segment coding for the FLAG octapeptide (IBI/Kodak) at C terminal was amplified by PCR and inserted into the mammalian expression vector pcDNA3 (Invitrogen) under the control of the cytomegalovirus promoter (construct LM357). CHO cells were transfected with LM357 by using calcium phosphate precipitation. Stable clones were selected in DMEM containing 10% FCS and 800 μg/ml G418. To assay the presence of Figf/Vegf-D in CHO supernatants, isolated clones were grown in DMEM containing 2% FCS and 800 μg/ml G418 and analyzed by ELISA using anti-Figf/Vegf-D rabbit polyclonal antiserum (15). Supernatant from positive clones was precipitated with deoxycholate acid and analyzed by Western blot. Different CHO clones expressed different Figf/Vegf-D levels. Specifi-

Abbreviations: Figf, c-*fos*-induced growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; HUVEC, human umbilical cord vein endothelial cell; KS-IMM, Kaposi's sarcoma-immortal; CHO, Chinese hamster ovary.

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cally, clone 65 expressed less than 0.1 ng/ml of Figf/Vegf-D in the cell supernatant *in vitro* whereas clone 79 expressed approximately 0.5 ng/ml of Figf/VEGF-D in the same conditions.

To express Figf/Vegf-D in yeast a cDNA fragment encoding the portion of the mouse Figf/Vegf-D polypeptide from residues 91 to 208 with six histidine residues at N terminus was amplified by PCR and inserted into the expression vector Yepsec1 immediately downstream from DNA sequence encoding the *Kluyveromyces lactis* toxin leader peptide (LM375) (30). The protein was expressed in *Saccharomyces cerevisiae* yeast strain by adding galactose to the yeast culture medium because Yepsec1 construct contains a galactose upstream activation sequence and the 5′ nontranslated leader of the yeast CYC1 gene, up to position −4 from the ATG translation initiation codon (30). Figf/Vegf-D glycosilation mutant was obtained by PCR with the substitution N160P (LM376). Figf/Vegf-D and Figf/Vegf-D N160P proteins were purified from the yeast supernatant by using a nickel column (HiTrap Chelating columns Pharmacia Biotech) under native conditions.

***In Vivo* Angiogenic Assay.** The angiogenic activity of Figf/Vegf-D was assayed *in vivo* by using the rabbit cornea assay previously described (31). Corneal assays were performed in male New Zealand albino rabbits (Charles River, Calco, Lecco, Italy) in accordance with the guideline of the European Economic Community for Animal Care and Welfare (EEC Law No. 86/609). Briefly, after being anaesthetized with sodium pentotal (30 mg/Kg), a micro pocket (1.5 × 3 mm) was surgically produced by using a pliable iris spatula 1.5 mm wide in the lower half of the cornea. The cell suspension (from 2.5 to 4 × 10^5 cells/5 ml) or slow-release pellets of Elvax-40 (DuPont) containing the purified growth factor were implanted into the micro pocket. Subsequently daily observation of the implants was made with a slit lamp stereomicroscope without anesthesia. An angiogenic response was scored positive when budding of vessels from the limbal plexus occurred after 4 days and capillaries progressed to reach the implanted pellet according to the scheme previously reported (32). The potency of angiogenic activity was evaluated on the basis of the number and growth rate of newly formed capillaries, and an angiogenesis score was calculated as described (32). Corneas were removed at the end of the experiment as well as at defined intervals after surgery and/or treatment and fixed in formalin for histological examination. A minimum of four independent experiments was performed for each condition.

Cell Cultures. Human endothelial cells were isolated from umbilical cord vein by collagenase treatment as described (33) and used at passage 1–4. KS-IMM cells were derived from a non-AIDS patient and are immortalized without signs of senescence after more than 120 *in vitro* passages. This cell line shares common markers and similar biological behavior with typical KS "spindle cells" (34). Cells were grown on gelatin-coated plastic, in medium M 199 supplemented with 20% heat-inactivated FCS, penicillin (100 units/ml), streptomycin (50 μg/ml), heparin (50 μg/ml), and bovine brain extract (100 μg/ml) (Life Technologies, Milan, Italy).

***In Vitro* Angiogenesis.** Because Matrigel can induce spontaneously *in vitro* angiogenesis, we tested more preparations and used batches devoid of this activity. Fifty microliters of Matrigel (Collaborative Research, lot 901448) (35) was added per well of 96-well tissue culture plates and allowed to gel at 37°C for 10 min. HUVECs were starved for 24 h in M199 with 1% FCS before being harvested in PBS-EDTA. Cells (10^4) were gently added to each of triplicate wells and allowed to adhere to the gel coating for 30 min at 37°C. Then, medium was replaced with indicated concentrations of Figf/Vegf-D. The plates were monitored after 24 h and photographed with a Canon microscope. Each experiment was repeated at least three times with identical results.

Immunoprecipitation and Western Blotting. Subconfluent cultures were starved as above and then cells were stimulated with the indicated concentrations of Figf/Vegf-D for 10 min at room temperature. Positive control was done by incubating cells with sodium orthovanadate (0.1 mM H_2O_2, 1 mM Na_3OV_4) for 20 min at 37°C. After three washes with cold PBS containing 1 mM sodium orthovanadate, cells were lysed for 20 min on ice in 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM Ne_3VO_4, 1 mM PMSF, 0.1 mM $ZnCl_2$, 1% Triton. Lysates (1 mg of total proteins) were incubated at 4°C for 2 h with 100 μl of a 50% solution of protein A-Sepharose (Amersham-Pharmacia Biotech) in 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, and anti-VEGFR-2 (Santa Cruz Biotechnology, sc-504) or anti-VEGFR-3 (Santa Cruz Biotechnology, sc-321). Immunoprecipitates were washed four times with lysis buffer and analyzed by 8% SDS/PAGE. Proteins were transferred onto a nylon membrane [poly(vinylidene difluoride), Millipore] and analyzed by immunoblotting with antiphosphotyrosine mAb (Upstate Biotechnology, Lake Placid, NY). Staining was performed by a chemiluminescence assay (ECL, Amersham-Pharmacia Biotech).

Cell Growth Assay. HUVECs (2.5 × 10^3) or KS-IMM cells were plated in 96-well plates (Costar) coated with gelatin (Difco; 0.05%, for 1 h at 22°C) in M199 medium containing 20% FCS (Irvine Scientific). After 24 h the medium was removed and replaced with M199 containing 1% FCS with or without Figf/Vegf-D; fresh factor was added every 2 days. Endothelial cell numbers were estimated after staining with crystal violet by a colorimetric assay described by Keung *et al.* (36).

Chemotaxis Assay. Chemotaxis assays on HUVECs and KS-IMM were performed as described (33, 37) with the Boyden chamber technique using a 48-well micro chemotaxis chamber. Polyvinylpyrrolidone-free polycarbonate filters (Nucleopore, Corning-Costar) with a pore size of 5 μm were coated with 1% gelatin for 10 min at room temperature and equilibrated in M199 supplemented with 1% FCS. Indicated concentrations of purified Figf/Vegf-D were placed in the lower compartment of a Boyden chamber. Subconfluent cultures were starved as above, harvested in PBS (pH 7.4) with 10 mM EDTA, washed once in PBS, and resuspended in M199 containing 1% FCS, at a final concentration of 2.5 × 10^6 cells/ml. After placing the filter between the lower and upper chambers, 50 μl of the cell suspension was seeded in the upper compartment. Cells were allowed to migrate for 7 h at 37°C in a humidified atmosphere with 5% CO_2. The filter then was removed, and cells on the upper side were scraped with a rubber policeman. Migrated cells were fixed in methanol, stained with Giemsa solution (Diff-Quick, Baxter Diagnostics, Rome) and counted from five random high-power fields (magnification ×100) in each well. Each experimental point was studied in triplicate.

RESULTS

Induction of Angiogenesis *in Vivo*. Mature VEGF-C and Figf/VEGF-D factors are generated by proteolytic cleavages of both of the N- and C-terminal domains during secretion (15, 23, 38). To obtain recombinant mature Figf/Vegf-D we generated CHO clones by stable transfection of constructs containing the mouse Figf/Vegf-D cDNA truncated at the C-terminal proteolytic site (38). To assess *in vivo* the angiogenic activity of increasing concentrations of the recombinant protein administered to avascular tissue two clones expressing different levels of secreted Figf/Vegf-D were selected for implantation into rabbit corneas. These clones secrete in the culture medium Figf/Vegf-D in two main forms of molecular mass of 30 and 21 kDa, respectively (Fig. 1*A*). Both clone 65 and clone 79 induced corneal vascularization whereas the CHO mock transfectant clone did not show any angiogenic

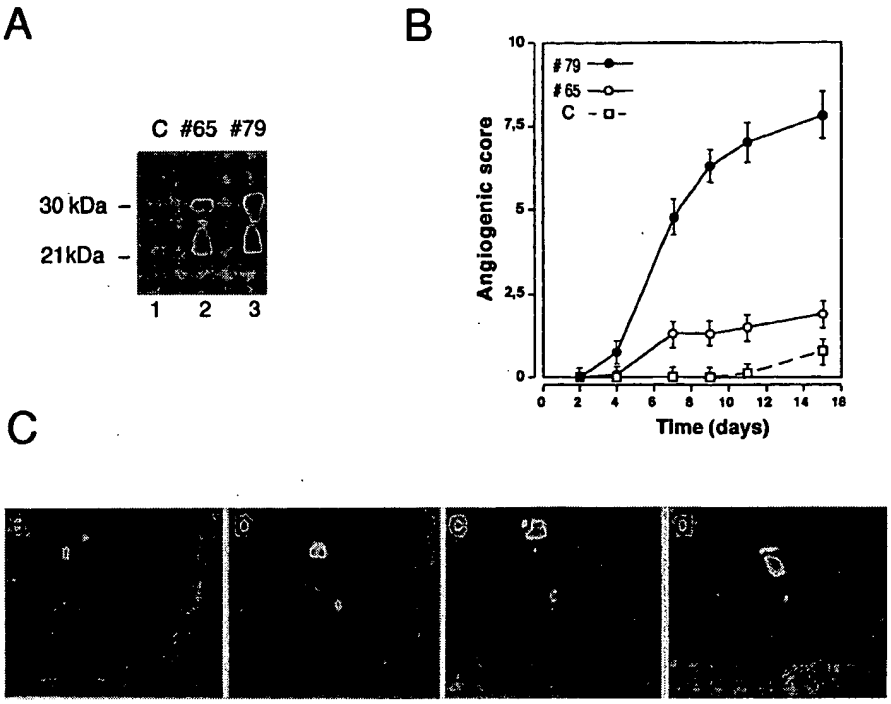

FIG. 1. Implanted Figf/Vegf-D-expressing cells induce neovascularization in rabbit corneas. (*A*) Figf/Vegf-D expressed in CHO cells. Equal volumes of culture supernatants from clones 65 and 79 were precipitated and analyzed by Western blot using an anti-Figf/Vegf-D rabbit polyclonal antiserum. (*B*) CHO cells (4×10^4) expressing Figf/Vegf-D were surgically implanted into the corneas. New blood vessel growth was recorded every other day with a slit lamp stereomicroscope. Angiogenic scores were calculated on the basis of the number of vessels and their growth rate and plotted versus time (for experimental details see *Materials and Methods*). Angiogenic score data are the mean values obtained from the response scored in all animals in this study. C, CHO mock transfectant clone; #65, clone expressing low levels of Figf/Vegf-D (0.1 ng/ml protein in supernatant); #79 clone expressing higher levels of Figf/Vegf-D (approximately 0.5 ng/ml protein in supernatant). (*C*) Pictures of rabbit corneas from a representative experiment. (*a*) Corneal implant of CHO mock transfectant. Clone 79 promotes and sustains vascular growth over time at day 6 (*b*), 9 (*c*), and 14 (*d*). Corneas were photographed with a stereomicroscope. Magnification: ×18.

effect (Fig. 1*B*). Although a direct dose response could not be made in this assay, the efficiency of the angiogenic response correlated with the amount of growth factor released *in vitro* as clone 79 secreted about 5-fold more Figf/Vegf-D than clone 65 in the same conditions (Fig. 1*A*). Consistently, neovascular growth induced by clone 79 was more efficient and persisted in 100% of the implants whereas clone 65 did so in only 30% of corneas (Fig. 1*B*). This angiogenic activity also was suggested by the direct correlation between neovascular growth observed and the number of cells implanted into corneal micro pocket (data not shown). The angiogenic response obtained with clone 79 (Fig. 1*C*) was comparable to the one elicited with cells expressing VEGF-A_{121} (39) both in intensity and appearance.

To obtain larger amounts of pure Figf/Vegf-D it also was expressed in yeast *S. cerevisiae*. To obtain a secreted Figf/Vegf-D form in yeast supernatants the cDNA fragment encoding the portion of the mouse Figf/Vegf-D polypeptide from residues 91 to 208 plus a segment coding for six histidine residues at the N terminus was cloned in a yeast vector containing a secretion signal. This recombinant protein expressed in yeast was secreted into the culture medium (Fig. 2*A*). By contrast with the other members of the VEGF family, VEGF-C and Figf/Vegf-D contain two putative glycosylation sites in the mature protein. Secreted Figf/Vegf-D is glycosilated at asparagine-160 residue in both mammalian and yeast cells (data not shown). To test the activity of both the glycosylated and unglycosylated forms we also generated a Figf/Vegf-D mutant in which the glycosylation site was mutated by the introduction of a proline residue at position 160, which is present in all other known VEGF family members. Consistent with N-linked glycosylation, the wild-type protein shows about 2-kDa molecular mass increase with respect to the mutant Figf/Vegf-D N160P (Fig. 2*A*) and it is sensitive to endoglycosidase H (not shown).

Figf/Vegf-D purified to homogeneity was analyzed in the corneal micro pocket assay *in vivo*. Similar to the results obtained with implanted CHO cells, purified Figf/Vegf-D induced a strong angiogenic response. After the implant of a single dose of protein in the slow-release pellets all Figf/Vegf-D doses of 100–400 ng/pellet induced capillary growth after just 3 days. However, a clear effect of increasing Figf/Vegf-D concentration was evident at later time points (Fig. 2*B*). The Figf/Vegf-D N160P mutant showed less potent angiogenic activity with respect to the wild-type protein (Fig. 2*C*), suggesting that Figf/Vegf-D glycosylation is involved in receptor recognition. In this assay, recombinant Figf/Vegf-D showed intermediate activity when compared with VEGF-A_{121} and VEGF-A_{165} (Fig. 2*D*) when used at doses of 300–400 ng. Corneal angiogenesis induced by either Figf/Vegf-D or VEGF-A was noninflammatory (not shown).

Figf/Vegf-D Induces *in Vitro* Angiogenesis. Studying endothelial cell behavior in a three-dimensional culture system, consisting of extra cellular matrix proteins, allows for *in vitro* conditions that more closely mimic the *in vivo* environment permissive for cell differentiation into capillary-like structures. This assay system is called *in vitro* angiogenesis (40). To examine whether Figf/Vegf-D induces *in vitro* morphological changes resembling of capillary like-structure formation, endothelial cells were plated on a three-dimensional matrix of Matrigel (41) and then stimulated with increasing concentrations of Figf/Vegf-D. Endothelial cells grown under these conditions in the presence of 1% BSA exhibited a small round shape and did not spread. Treatment with Figf/Vegf-D for 24 h resulted in dramatic dose-dependent morphological changes. The cells became elongated, forming thin cords of intercon-

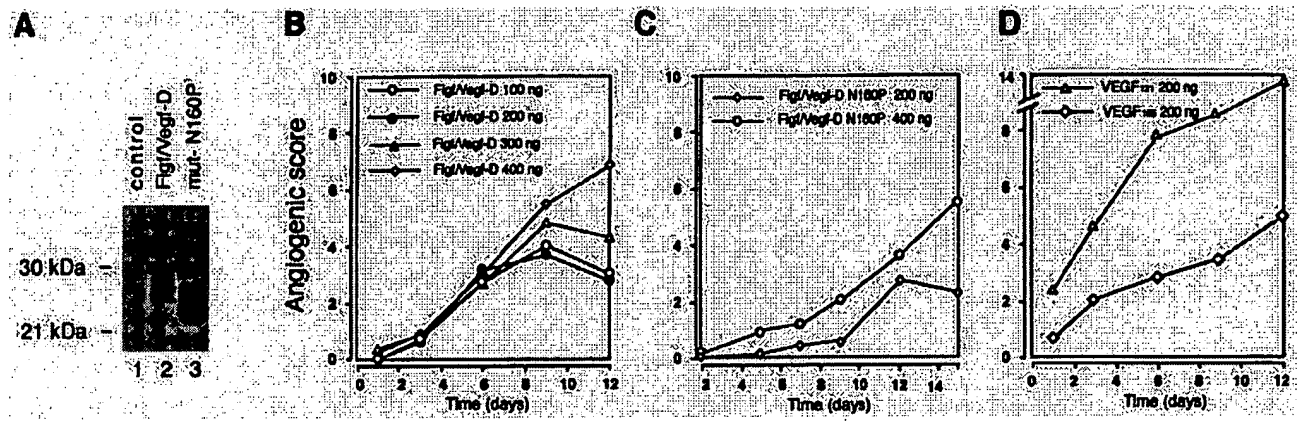

FIG. 2. Figf/Vegf-D sustains dose-dependent angiogenesis *in vivo*. (*A*) Supernatant of *S. cerevisiae* yeast strains expressing Figf/Vegf-D and Figf/Vegf-D mutant as indicated. (*B*) The angiogenic activity of various concentrations of Figf/Vegf-D were tested as slow-release preparations in the rabbit cornea assay. (*C*) Angiogenic activity of 200 and 400 ng/pellet of Figf/Vegf-D N160P. (*D*) Angiogenic activity of 200 ng/pellet of VEGF-A_{121} and VEGF-A_{165} is shown for comparison. Angiogenic score data are the mean values obtained from the responses scored in all animals in this study. Variations were below 10% of the mean values. Angiogenic scores are calculated as described in Fig. 1 and in *Materials and Methods*.

necting cells (Fig. 3). The effect was investigated in a range of concentrations between 5 and 200 ng/ml and was maximal at 100 ng/ml. Similar effects also were observed with 20 ng/ml VEGF-A. These data demonstrate that Figf/Vegf-D, like VEGF-A, is able to mediate dramatic cell reorganization, which would be necessary *in vivo* for the sprouting of endothelial cells and tube formation. No morphological alterations could be observed in KS-IMM cells, either by treating the cells with VEGF-A or with Figf/Vegf-D (not shown).

Figf/Vegf-D Induction of VEGFR-2 and VEGFR-3 Tyrosine Phosphorylation. It has been reported recently that VEGF-D and VEGF-C are the ligands of the endothelial tyrosine kinase receptors VEGFR-2 and VEGFR-3 (14, 23). To examine the cellular response of endothelial cells to Figf/Vegf-D *in vitro*, we first tested whether Figf/Vegf-D could stimulate signal transduction from VEGFR-2 and VEGFR-3 in HUVECs and KS-IMM cells because these cells express both receptors. Tyrosine phosphorylation of these receptors was assayed in serum-starved cells treated with Figf/Vegf-D. VEGFR-2 and VEGFR-3 were immunoprecipitated with specific antibodies and analyzed by Western blotting with antiphosphotyrosine-specific antibodies. Figf/Vegf-D stimulated tyrosine phosphorylation of the 210-kDa VEGFR-2 and both the 125- and 195-kDa processed and unprocessed forms of VEGFR-3 in both HUVECs and KS-IMM cells (Fig. 4). Thus, Figf/Vegf-D, like VEGF-C, binds and activates these receptors on endothelial cells.

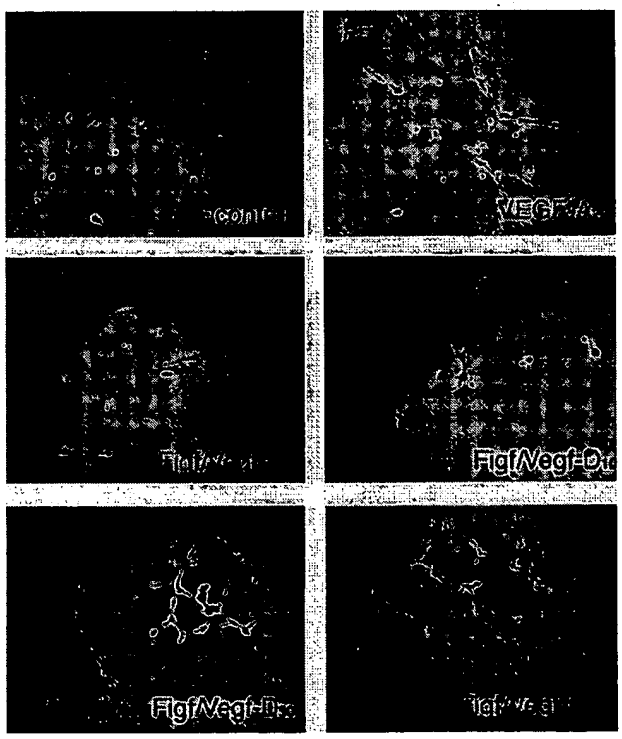

FIG. 3. Figf/Vegf-D-induced endothelial cell morphological changes. VEGF-A or Figf/Vegf-D were added to HUVECs cultured in three-dimensional Matrigel in low serum conditions. Photographs were taken 24 h after Figf/Vegf-D treatment. Protein concentrations (ng/ml) used are indicated.

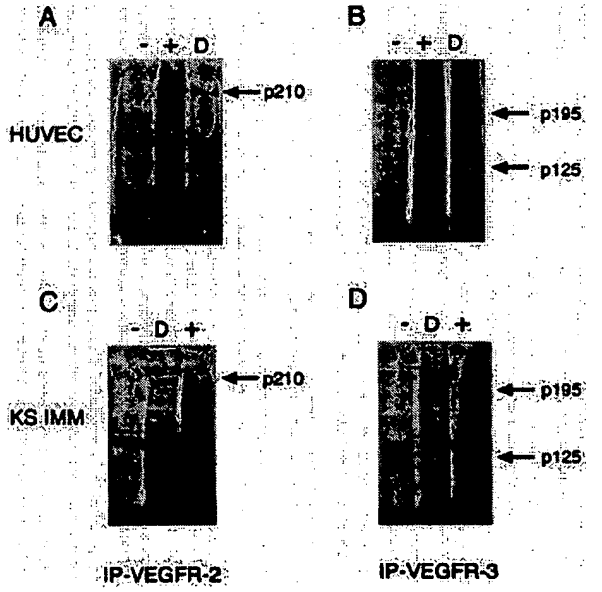

FIG. 4. Figf/Vegf-D-induced tyrosine phosphorylation of VEGFR-2 and VEGFR-3. HUVECs and KS-IMM cells were incubated with Figf/Vegf-D. After stimulation receptors were immunoprecipitated with antireceptor antibodies and analyzed by Western blotting with an antiphosphotyrosine mAb. (*A* and *B*) Phosphorylation of VEGFR-2 and VEGFR-3 in HUVECs. (*C* and *D*) Phosphorylation of VEGFR-2 and VEGFR-3 in KS-IMM cells. Positive control (+) and Figf/Vegf-D stimulation (D) is indicated. Arrows denote the position of the phosphorylated 210-kDa VEGFR-2 protein and the positions of the phosphorylated, proteolytically processed 125-kDa and unprocessed 195-kDa forms of VEGFR-3.

Figf/Vegf-D Induction of Growth and Chemotaxis in HUVECs and KS-IMM Cells. To investigate further the proliferative effect of Figf/VEGF-D on endothelial cells, we incubated both the cultured HUVECs and KS-IMM cells in the presence of increasing concentrations of Figf/Vegf-D. Proliferation of both cell types was stimulated in a dose-dependent manner (Fig. 5 *A* and *B*). The effect was investigated in a range of concentrations between 5 and 100 ng/ml and was maximal at 50 ng/ml for both cell types. Interestingly, when suboptimal concentrations of VEGF-A_{165} and Figf/Vegf-D were coadded to HUVECs the resulting proliferation was higher than the treatment of each alone (not shown).

The chemotactic effect of Figf/Vegf-D on HUVECs and KS-IMM cells was analyzed in a modified Boyden chamber assay. The migration of the cells through collagen-coated micropore filters toward chemoattractants was scored in the absence of serum. Figf/Vegf-D stimulated the migration of KS-IMM cells in a dose-dependent manner. In HUVECs, under identical conditions Figf/Vegf-D induced little or no migration (Fig. 5 *C* and *D*).

DISCUSSION

The results reported in this work show that Figf/Vegf-D is a potent angiogenic factor. In rabbit corneas Figf/Vegf-D, expressed either in CHO or yeast cells, can efficiently induce angiogenesis. The dose dependency and the early response suggest a direct effect of Figf/Vegf-D on endothelial cell recruitment *in vivo*. This observation has been confirmed by *in vitro* experiments that show direct Figf/Vegf-D activities on endothelial cells. In addition to Figf/Vegf-D, other factors, including VEGF-A, basic fibroblast growth factor, placental growth factor, VEGF-C, and VEGF-E induce angiogenesis, suggesting a redundancy or a coordination among factors performing the same function (2, 10, 42, 43). The generation of VEGF-A knockout mice demonstrated that this factor is essential for angiogenesis during development (6, 7). Thus, simple redundancy of all members of the family is unlikely. We favor the hypothesis that the complex process of angiogenesis normally requires the cooperation of multiple factors and the experimental overexpression of some key members is able to trigger the process both directly and indirectly, inducing the expression of other factors. Each of these factors shows a peculiar pattern of expression, suggesting that a complex balance of factors in different developing organs may be relevant. Moreover, the biological function of VEGFs may not be limited to angiogenesis. For instance, fibroblast growth factors not only induce angiogenesis, but are also regulators of embryonic development, influencing the formation of several structures including body axis, limbs, heart, and lung differentiation (44–47). Similarly, the expression of Figf/Vegf-D in tissues like the pituitary, the developing teeth, lung mesenchyme, and limb buds (26) suggests that Figf/Vegf-D, in addition to playing a role in angiogenesis, could be involved in specific inductive signaling in these developing organs.

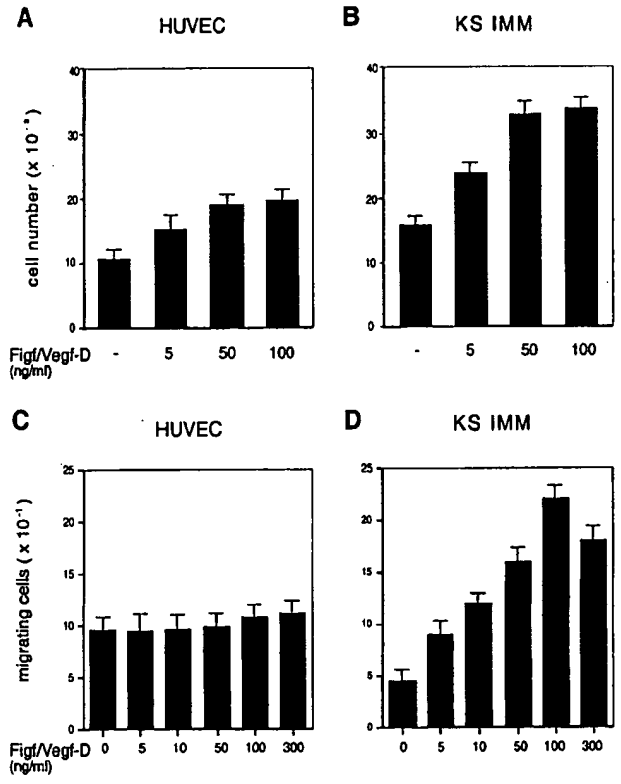

FIG. 5. Figf/Vegf-D-induced cell proliferation and chemotactic activity. (*A* and *B*) Proliferative effects of Figf/Vegf-D were assayed on HUVECs and KS-IMM cells as indicated. Experiments were performed in medium containing 1% FCS. After 72 h cells were enumerated by using a Coulter counter and values represent the mean (±SEM) of triplicate samples. (*C* and *D*) Cells were seeded in the upper wells of a 48-well micro chemotaxis Boyden chamber and incubated for 7 h at 37°C in medium containing 1% FCS. The lower wells contained the indicated concentrations of Figf/VEGF-D. Cells migrating through a polycarbonate membrane with a pore size of 5 μm were quantified by staining the cells with Giemsa solution and counting was performed on a light microscope of five high-power fields (×100). The results are expressed as the mean ± 1 SD of three independent experiments performed in triplicate.

Figf/Vegf-D and VEGF-C share striking similarities in their primary sequence and posttranslational modifications, and most importantly, both factors are recognized by VEGFR-2 and VEGFR-3 present on vascular and lymphatic vessels (14, 23). By using porcine aortic endothelial cells selectively overexpressing VEGFR-2 or VEGFR-3, it was shown recently that VEGF-C could promote migration and proliferation independently of signaling through either receptor (21). In this study for the analysis of Figf/Vegf-D activity *in vitro* we used HUVECs and KS-IMM cells, which express both receptors. By immunoprecipitation experiments we showed that Figf/Vegf-D activates both VEGFR-2 and VEGFR-3 tyrosine phosphorylation on both HUVECs and KS-IMM cells. The activation of these receptors by Figf/Vegf-D stimulates a biological response that involves morphological, mitogenic, and motogenic responses. Thus, Figf/Vegf-D shows a direct activity on endothelial cells *in vitro*, confirming the *in vivo* data. Although both HUVECs and KS-IMM cells show a similar mitogenic response to Figf/Vegf-D they differ in motogenic responses because KS-IMM cells are more responsive than HUVECs to motogenic activation by Figf/Vegf-D. This discrepancy could be simply because of differences in the receptor levels or in intracellular signaling molecules, but it also could be the result of the presence of possible coreceptors that may modify the receptor affinity and modulate the response to Figf/Vegf-D. In line with this possibility, it recently has been shown in human endothelial cells that neuropilin-1 (48) and $\alpha v\beta 3$ modulate the activity of VEGFR-2. Neuropilil-1 is a coreceptor for the VEGF-A_{165} isoform and the $\alpha v\beta 3$ integrin associates with VEGFR-1 upon VEGF-A stimulation and regulates the level of tyrosine phosphorylation of the receptor (49).

Figf/Vegf-D differs from all other members of the VEGF family because it is the only angiogenic factor regulated by the nuclear oncogene c-*fos* (15). This unique regulation of Figf/Vegf-D may be relevant both during development and in tumor progression because c-*fos* is involved not only in transformation but also in the regulation of cell growth and differentiation of various tissues (50, 51). Tumors that develop in c-*fos*-deficient mice appear devoid of vascularization although, in

these papillomas, VEGF-A expression is reduced but not absent (52). The role of Figf/Vegf-D in tumor progression is at the moment unclear, although the evidence that Figf/Vegf-D shows angiogenic activity on endothelial cells, as well as mitogenic and motogenic activity on tumor-derived KS-IMM cells, strongly suggest that Figf/Vegf-D can be a *c-fos* effector for tumor malignancy.

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